Full Paper

μ-Opioid Receptor Forms a Functional Heterodimer With Cannabinoid CB1 Receptor: Electrophysiological and FRET Assay Analysis

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Abstract. Interactions between μ-opioid receptor (μOR) and cannabinoid CB1 receptor (CB1R) were examined by morphological and electrophysiological methods. In baby hamster kidney (BHK) cells coexpressing μOR fused to the yellow fluorescent protein Venus and CB1R fused to the cyan fluorescent protein Cerulean, both colors were detected on the cell surface; and fluorescence resonance energy transfer (FRET) analysis revealed that μOR and CB1R formed a heterodimer. Coimmunoprecipitation and Western blotting analyses also confirmed the heterodimers of μOR and CB1R. [D-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) or CP55,940 elicited K+ currents in Xenopus oocytes expressing μOR or CB1R together with G protein activated-inwardly rectifying K+ channels (GIRKs), respectively. In oocytes coexpressing both receptors, either of which was fused to the chimeric Ga protein Gqi5 that activates the phospholipase C pathway, both DAMGO and CP55,940 elicited Ca2+-activated Cl− currents, indicating that each agonist can induce responses through Gqi5 fused to either its own receptor or the other. Experiments with endogenous Gi/o protein inactivation by pertussis toxin (PTX) supported the functional heterodimerization of μOR/CB1R through PTX-insensitive Gqi5(m) fused to each receptor. Thus, μOR and CB1R form a heterodimer and transmit a signal through a common G protein. Our electrophysiological method could be useful for determination of signals mediated through heterodimerized G protein–coupled receptors.

Keywords: μ-opioid receptor, cannabinoid CB1 receptor, receptor heterdimerization, fluorescence resonance energy transfer (FRET), electrophysiology

Introduction

G protein–coupled receptors (GPCR) were previously considered to have monomeric structures. However, recent evidence suggests that some or even most of the GPCR are oligomeric structures formed by GPCR homodimers, heterodimers, multimers, and also different types of proteins that intercommunicate at the plasma membranes (1 – 3). It is well known that opioids that bind to G protein–coupled μ-opioid receptors (μOR) and cannabinoids that bind to G protein–coupled cannabinoid receptors (CB1R) expressed in the central nervous system have common pharmacological effects, such as antinociception, hypothermia, inhibition of locomotor activity, hypotension, and sedation (4, 5). The synergy in the analgesic effects of opioids and cannabinoids is attributed to a cross-talk between these two signaling pathways mediated by simultaneous activation of opioid and cannabinoid receptors. Furthermore, delta 9-tetrahydrocannabinol, a cannabinoid CB1R agonist, can enhance the potency of opioids such as morphine (6). Recent studies reported that μOR and CB1R form a heterodimer in the heterologous expression system (7). However, confirmation of direct functional signaling through such heterodimerized receptors remains to be elucidated.

In the present study, several experiments were conducted to confirm the heterodimerization of μOR and
CB1R. Morphological analysis was employed using fluorescence resonance energy transfer (FRET) assay and baby hamster kidney (BHK) cells expressing receptors fused to fluorescent proteins. Whether μOR and CB1R form heterodimers was determined using BHK cells expressing CB1R fused to Cerulean, a brighter variant of cyan fluorescent protein (CFP) (8) and μOR fused to Venus, a brighter variant of yellow fluorescent protein (YFP) (9). We also conducted coimmunoprecipitation with subsequent western blot assay in BHK cells expressing FLAG-tagged μOR and CB1R. Electrophysiological assay was also conducted to confirm the formation of functional heterodimers of μOR and CB1R. To this end, we developed an electrophysiological assay using the Xenopus oocyte expression system with μOR and CB1R fused to a chimeric Gq5 protein, Gq5i. The chimeric Gq5i protein, whose last five amino acid residues in the C-terminus was replaced with the corresponding portion of G1 protein, is useful for monitoring the responses mediated by stimulation of Gi/o protein-coupled receptors in the Xenopus oocyte expression system, as reported previously by our laboratory (10, 11). Chimeric Gq5i allows Gi/o-coupled receptors to couple to the phospholipase C (PLC)-mediated signal pathway (12, 13). Agonists for Gi/o protein-coupled receptors can elicit Ca2+-activated Cl− currents in such oocytes only through the chimeric Gq5i, but not through Gi/o endogenously expressed in oocytes (10, 11). Based on the results, we proposed that our electrophysiological method for functional analyses could be useful for determination of signals mediated through heterodimerized G protein–coupled receptors.

Materials and Methods

Drugs and chemicals
Baclofen, gentamicin, sodium pyruvate, [d-Ala²,N-Me-Phe³,Gly⁵-ol]enkephalin (DAMGO), and pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO, USA). CP55,940 was from Tocris Bioscience (Bristol, UK). Other chemicals used in the study were of analytical grade and obtained from Nacalai Tesque (Kyoto).

Construction of cDNAs and preparation for cRNA
cDNAs for the rat μOR was kindly provided by Dr. N. Dascal (Tel Aviv University, Ramat Aviv, Israel), human CB1R was from Dr. M. Parmentier (Université Libre de Bruxelles, Belgium), and rat G protein activated-inwardly rectifying K+ channel 1 (GIRK1) and mouse GIRK2 were donated by Dr. H.A. Lester (Caltech, Pasadena, CA, USA). GABA𝛼3R and GABA𝛼5R subunits were from Dr. N. Fraser (Glaxo Wellcome, UK). Cerulean, a brighter variant of CFP (8) was from Dr. D.W. Piston (Vanderbilt University, TN, USA), and Venus, a brighter variant of YFP (9) was from Dr. T. Nagai (Hokkaido University, Sapporo). The chimeric Gq5i was a kind gift from Dr. B.R. Conklin (University of California at San Francisco, CA, USA). PTX-insensitive chimeric Gq5i protein Gq5i(m), whose cysteine residue at −4 from the C-terminus end was changed to isoleucine, was created with a site-directed mutagenesis kit (QuickChange TM site-directed mutagenesis Kit; Stratagene, Tokyo). The CB1R-Cerulean/Venus, μOR-Cerulean/Venus, and GABA3R-Cerulean/Venus were generated by ligating the receptor cDNAs into NotI or BamHI sites into the corresponding site of Venus or Cerulean cDNAs. The tandem cDNAs of μOR-Gq5i/Gq5i(m), CB1R-Gq5i/Gq5i(m), and GABA3R-Gq5i/Gq5i(m) were created by ligating the receptor cDNA sequences into the NheI site of Gq5i or Gq5i(m) cDNAs. FLAG-tagged μOR was constructed by PCR with the 5’-FLAG sequence–containing open reading frame primer and the 3’-end of the receptor sequence primer. The sequences of all PCR products were confirmed by sequencing with ABI3100 (Applied BioSystems, Tokyo). All cDNAs for transfection in BHK cells were subcloned into pcDNA3.1 (Invitrogen, Tokyo). All cDNAs for the synthesis of cRNAs were subcloned into the pGEMHJ vector, which provides the 5’- and 3’-untranslated region of the Xenopus β-globin RNA (14), ensuring a high level of protein expression in the oocytes. Each of the cRNAs was synthesized using the mCAP mRNA Capping Kit, with T7 RNA polymerase in vitro Transcription Kit (Ambion, Austin, TX, USA) from the respective linearized cDNAs.

Cell culture and transfection
BHK cells were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere at 95% air and 5% CO2. For transfection experiments, BHK cells were seeded at a density of 1 × 105 cells/35-mm glass-bottomed culture dish (World Precision Instrument, Sarasota, FL, USA) for 24 h. For western blot assay, cells were seeded at a density of 1 × 106 cells/35-mm dish. Transient transfection was then performed with Effectene transfection reagent (Qiagen, Tokyo) containing 0.2 μg each of cDNAs as described previously (11, 15). Cells were used in confocal microscopy analysis, FRET analysis, and Western blotting assay 16 – 24 h after transfection.

Confocal microscopy for FRET analysis
For the analysis of heterodimerization of CB1R and
μOR with FRET assay, CB1R, μOR, and GABA\textsubscript{B}Rs were fused at their carboxy terminus to Cerulean or Venus. Both Cerulean and Venus were excited with 458-nm laser, and images were obtained by placing the dish onto a stage in a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany).

Photobleaching and calculation of FRET efficiency

To confirm FRET between Cerulean and Venus, we monitored acceptor photobleaching analysis in BHK cells that coexpressed Cerulean- and Venus-fused receptors. FRET was measured by imaging Cerulean before and after photo-bleaching Venus with the 100% intensity of 514-nm argon laser for 30–60 s, a duration that efficiently bleached Venus with little effect on Cerulean (11, 15). An increase of the donor fluorescence (Cerulean) was interpreted as evidence of FRET from Cerulean (11, 15). An increase of donor fluorescence.

Certo preparation and injection

Immature V and VI oocytes from *Xenopus* were enzymatically dissociated as described previously (18, 19). Isolated oocytes were incubated at 18°C in ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4) containing 2.5 mM sodium pyruvate and 50 μg/ml gentamicin. For measurement of GIRK currents induced by DAMGO or CP55,940, cRNAs for GIRK1/2 (0.2 ng each) were coinjected into the oocytes together with or without μOR, CB1R, GABA\textsubscript{B}1aR, μOR-Ven/HerCerulean, or CB1R-Cerulean/Ven (5 ng each). For the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current assay, cRNA for G\textsubscript{q5} and G\textsubscript{q5(m)}-fused μOR, CB1R, or GABA\textsubscript{B}R were injected. The final volume of the injectate was less than 50 nl in all cases. Oocytes were incubated in ND-96 and used 3–8 days after injection as reported previously (11, 18, 19).

Electrophysiological recordings

Electrophysiological recordings were performed using the two-electrode voltage clamp method with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA) at room temperature. Oocytes were clamped at −60 mV and continuously superfused with ND-96 or 49 mM high K\textsuperscript{+} (HK) solution (48 mM NaCl, 49 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 5 mM HEPES, pH 7.4) in a 0.25 ml chamber at a flow rate of 5 ml/min, and test compounds were added to the superfusate. Voltage recording microelectrodes were filled with 3 M KCl and their tip resistance was 1.0–2.5 MΩ. Currents were continuously recorded and stored with a MacLab (AD Instruments, Castle Hill, NSW, Australia) and a Macintosh computer, as described previously (18, 19). All test compounds applied to oocytes were dissolved into ND-96 or HK (49 mM K\textsuperscript{+}) medium.
**Statistical analyses**

Data are expressed as mean ± S.E.M. Differences between two groups were examined for statistical significance by using the paired t-test. For comparisons between multiple groups, one-way analysis of variance (ANOVA) was used followed by Scheffe’s test. GraphPad Prism (GraphPad, La Jolla, CA, USA) software was used for data analysis. A P value of less than 0.05 denoted the presence of a statistically significant difference.

**Results**

**FRET and acceptor bleaching analysis of Venus-fused µOR and Cerulean-fused CB₁R expressed in BHK cells**

In order to confirm FRET, we performed acceptor photobleaching analysis (Fig. 1). As a control study, we constructed CB₁R tandemly fused to both Cerulean and Venus (CB₁R-Cerulean-Venus) and expressed it in BHK cells. As shown in the upper panels of Fig. 1A, fluorescence from Cerulean and Venus was detected by...
scanning while exciting with the 458 nm laser line. The use of acceptor photobleaching increased Cerulean fluorescence (donor) and decreased Venus fluorescence (acceptor) (Fig. 1A, lower panel, and Fig. 1B). As shown in Fig. 1C, FRET efficiency was significantly higher in BHK cells expressing CB₁R-Cerulean-Venus, CB₁R-Venus-Cerulean, and µOR-Cerulean-Venus than in the negative control (Cerulean + Venus).

As shown in the upper panels of Fig. 2A, fluorescence of Venus and Cerulean was observed in the cell membrane and cytoplasm of BHK cells coexpressing µOR-V + CB₁R-C, at pre-photobleaching (Fig. 2A). The photo-bleaching results showed that Cerulean fluorescence increased with a decrease in Venus fluorescence in both the cell membranes and the cytosol (Fig. 2A, lower panel). As shown in Fig. 2B, the FRET efficiency was significantly higher in BHK cells coexpressed with µOR-V + CB₁R-C compared with the negative control [pairs of Venus (V) + Cerulean (C), µOR-V + GABA₁R-C or CB₁R-C + GABA₁R-V], as well as a heterodimer pair of GABA₁R-V/C, known to form an obligatory heterodimer to be functional (11). When the FLAG-tagged µOR-V was coexpressed with CB₁R-C in BHK cells, the FRET efficiency was not different from BHK cells expressed non-FLAG-tagged µOR-V (Fig. 2B).

**Coimmunoprecipitation and Western blotting of FLAG-µOR and CB₁R**

Immunoprecipitation with the anti-FLAG was performed with proteins extracted from BHK cells. Samples immunoprecipitated with FLAG antibody were blotted with anti-FLAG, anti-µOR, and anti-CB₁R antibodies (Fig. 3, left). The immune complexes blotted with anti-FLAG were identified in precipitants containing FLAG-tagged µOR (Fig. 3, top left). Furthermore, immune complexes blotted with anti-µOR were identified in precipitants containing FLAG-µOR (Fig. 3, middle left). The immune complexes blotted with anti-CB₁R antibody were found in precipitants containing CB₁R and FLAG-tagged µOR, but not CB₁R alone (Fig. 3, bottom left).

In experiments using total cell lysates, the immune complexes blotted by anti-CB₁R antibody were detected in CB₁R-expressing lysates such as in CB₁R alone or in combination of CB₁R with FLAG-µOR (Fig. 3, right).

**Responses to DAMGO or CP55,940 in Xenopus oocytes expressing µOR or CB₁R together with GIRK1 and GIRK2 (GIRK1/2)**

A selective µOR agonist DAMGO or a selective CB₁R agonist CP55,940 elicited inward rectifying K⁺ currents in oocytes coexpressing µOR or CB₁R together with GIRK1/2, respectively (Fig. 4: A and B) in a

![Fig 2](image-url). Confocal imaging and FRET analysis of the Venus-fused µOR (µOR-V) and Cerulean-fused CB₁R (CB₁R-C) coexpressed in a BHK cell. A: Visualization of the µOR-V and CB₁R-C in a cotransfected BHK cell and Cerulean and Venus fluorescences by photobleaching (after 30-s sustained application of 514-nm wavelength laser, lower panel). Fluorescence readings were performed 24 h after transfection as described in Materials and Methods. Calibration bar = 10 µm. B: Summary of FRET efficiency for the pair µOR-V and CB₁R-C and the pair FLAG-µOR-V and CB₁R-C. The pair GABA₁R-V + GABA₁R-C and the pair µOR-V + GABA₁R-C or CB₁R-C + GABA₁R-V were used as a positive and negative control for interacting receptors, respectively. Each column represents the mean ± S.E.M. of the FRET efficiency from independent experiments of 6 BHK cells.
concentration-dependent manner (Fig. 4C). On the other hand, no responses were recorded to DAMGO or CP55,940 in oocytes coexpressing μOR or CB₁R without GIRK1/2 (Fig. 4: A and B). The EC₅₀ of DAMGO was 10⁻⁷ M in oocytes expressing μOR with GIRK1/2 and that of CP55,940 was 3 × 10⁻⁷ M in oocytes expressing CB₁R with GIRK1/2.

Responses to DAMGO or CP55,940 in Xenopus oocytes expressing μOR fused to Gₐq/5 or CB₁R fused to Gₐq/5
Electrophysiological experiments were performed in oocytes expressing μOR and CB₁R, either of which was fused to Gₐq/5 protein. Since the chimeric Gₐq/5 protein contains the carboxyl-terminal five amino acids of Gα₃, the signal mediated by Gₛ/₁- or Gₛ/₅-coupled receptors is transferred to a PLC/IP₃/Ca²⁺-mediated signal, and thus oocytes expressing Gₐq/5-fused receptors can elicit Ca²⁺-activated Cl⁻ currents in response to each agonist (10–13). As expected, oocytes expressing Gₐq/5-fused μOR (μOR-Gₐq/5p) or Gₐq/5-fused CB₁R (CB₁R-Gₐq/5p) elicited Ca²⁺-activated Cl⁻ currents in response to DAMGO or CP55,940, respectively (Fig. 5: A and B) in a concentration-dependent manner (Fig. 5C). The EC₅₀ of DAMGO was ~10⁻⁷ M in oocytes expressing μOR-Gₐq/5 and that of CP55,940 in oocytes expressing CB₁R-Gₐq/5 was ~3 × 10⁻⁷ M. There were almost no differences in EC₅₀ of DAMGO and CP55,940 between oocytes expressing each receptor-Gₐq/5 and oocytes coexpressing each receptor with GIRK1/2 (Figs. 4 and 5), as reported previously (10).

Responses to DAMGO and CP55,940 in Xenopus oocytes coexpressing μOR and CB₁R either of which was fused to Gₐq/5
To investigate whether μOR and CB₁R can form a functional heterodimer, we coexpressed receptor-Gₐq/5 and the non-fused receptor counterpart in the oocytes. DAMGO and CP55,940 were sequentially applied to the oocytes and vice versa. Oocytes coexpressing non-fused μOR + CB₁R-Gₐq/5 elicited Ca²⁺-activated Cl⁻ currents in response to the 1st and 2nd application of DAMGO and CP55,940 (Fig. 6: A and D). In turn, when CP55,940 and DAMGO were sequentially applied to the oocytes coexpressing μOR + CB₁R-Gₐq/5, they elicited currents of almost the same size as the currents in oocytes applied with DAMGO followed by CP55,940 (Fig. 6: A and D). Similarly, oocytes coexpressing CB₁R + μOR-Gₐq/5 elicited Ca²⁺-activated Cl⁻ currents in response to the 1st application of CP55,940 followed by DAMGO and also to the 1st application of DAMGO followed by CP55,940; sizes of the currents of the 1st and 2nd application of each agonist were almost similar (Fig. 6D). These results show that both DAMGO and CP55,940, regardless of whether it was the 1st or 2nd sequential application, induced Ca²⁺-activated Cl⁻ currents in oocytes coexpressing μOR and CB₁R, either of which was fused to Gₐq/5 (Fig. 6: A, B and D). GABA₂Rs form obligatory functional heterodimers (11). As shown in Fig. 6C, when GABA₁₂B₁R and GABA₁₂B₂R-Gₐq/5 were expressed in oocytes, the GABA₁₂B₁R agonist baclofen elicited Ca²⁺-activated Cl⁻ currents, as reported previously (11). In oocytes coexpressing GABA₁₂B₁R + GABA₁₂B₂R-Gₐq/5 + μOR, baclofen but not DAMGO elicited the currents. Furthermore, in oocytes coexpressing GABA₁₂B₁R + GABA₁₂B₂R + μOR-Gₐq/5, DAMGO but not baclofen elicited Cl⁻ currents (Fig. 6C). No responses to DAMGO and CP55,940 were elicited in oocytes coexpressing μOR and CB₁R, both of which were not fused to Gₐq/5 (Fig. 6D).
Fig. 4. DAMGO- and CP55,940-induced GIRK currents in *Xenopus* oocytes coexpressing μOR and CB,R together with GIRK1/2 channels. A and B: Representative trace of each agonist-induced GIRK currents. Varying concentrations of DAMGO and CP55,940 were applied separately to the oocytes for 30 s as indicated by the bar. C: Concentration-response curves of DAMGO (open circle) and CP55,940 (closed circle) in oocytes expressing μOR or CB,R together with GIRK1/2. Varying concentrations of DAMGO and CP55,940 were applied separately to the oocytes for 30 s. Each point represents the mean ± S.E.M. of the peak GIRK currents expressed as percentage of the maximum response from 8 oocytes.

Fig. 5. DAMGO- and CP55,940-induced Cl⁻ currents in μOR-expressing oocytes fused to Gqi5 (μOR-Gqi5) and cannabinoid CB,R fused to Gqi5 (CB,R-Gqi5). A and B: Representative trace of each agonist-induced Ca²⁺-activated Cl⁻ current. C: Concentration-response curves of DAMGO (open circle) or CP55,940 (closed circle) in oocytes expressing μOR-Gqi5 or CB,R-Gqi5. Varying concentrations of DAMGO or CP55,940 were applied to the oocytes for 30 s. Each point represents the mean ± S.E.M. of the peak Cl⁻ currents expressed as a percentage of the maximum response from 8 oocytes.
µ-Opioid- and CB₁-Receptor Heterodimerization

Properties of transactivating responses induced by DAMGO or CP55,940 in oocytes coexpressing PTX-insensitive receptor-G₉₅₅

As shown in Fig. 6, both DAMGO and CP55,940 elicited Ca²⁺-activated Cl⁻ currents in oocytes coexpressing µOR-G₉₅₅ + CB₁R or µOR + CB₁R-G₉₅₅, suggesting functional heterodimer formation in oocytes. It has been reported that Gᵢₒ protein and G₉₅₅ are targets of ADP ribosylation of PTX at -4 cysteine from the C-terminus, and PTX treatment abrogates signals from Gᵢₒ-coupled receptors to Gᵢₒ as well as those to G₉₅₅ (11, 19 – 21). Another chimeric G₉₅₅₅₅₅, whose cysteine residue was changed to isoleucine, was reported to be insensitive to PTX (21). Accordingly, we pretreated oocytes coexpressing µOR + CB₁R-G₉₅₅ or µOR + CB₁R-G₉₅₅₅₅ with PTX (2 µg/ml) for 16 h (Fig. 7A). The concentra-
Fig. 7. Properties of DAMGO- and CP55,940-induced Cl⁻ currents in oocytes coexpressing μOR and CB₁R, one of which was fused to PTX-insensitive Gαqi₅(m) or PTX-sensitive Gαqi₅. Effects of DAMGO, CP55,940, or baclofen in oocytes coexpressing CB₁-R-Gαqi₅ or CB₁-R-Gαqi₅(m) with μOR (A), coexpressing μOR-Gαqi₅ or μOR-Gαqi₅(m) with CB₁R (B), or coexpressing GABAᵦ₂B₂R-Gαqi₅ or GABAᵦ₂B₂R-Gαqi₅(m) with GABAᵦ₁B₁aR (C). Oocytes were pretreated with PTX (0.2 mg/ml) for 16 h before electrophysiological measurement. In sequential application of DAMGO and CP55,940, DAMGO was first applied followed by CP55,940, and vice versa.

D: Summary of the effects of PTX treatment on Ca²⁺-activated Cl⁻ currents. Each bar represents the mean ± S.E.M. of peak Cl⁻ currents from each of 8 oocytes. *P<0.05, compared to the Cl⁻ currents in oocytes not pretreated with PTX. n.s: not significant compared to the currents in oocytes not pretreated with PTX.
tions and durations of PTX treatment used in the present study were reported to produce almost complete inactivation of the $G_{i/o}$ proteins in oocytes (11, 19). Under these conditions, oocytes coexpressing $\mu$OR + CB$_1$R-G$_{qi5}$ showed no responses to DAMGO or CP55,940, whereas both agonists caused $Cl^-$ currents in oocytes coexpressing $\mu$OR + CB$_1$R-$G_{qi5(m)}$ (Fig. 7: A and D). Similarly, oocytes coexpressing $\mu$OR-$G_{qi5(m)}$ + CB$_1$R responded to both DAMGO and CP55,940 pretreated with PTX (Fig. 7: B and D). In addition, PTX-pretreated oocytes coexpressing GABA$_{B2}$R + GABA$_{B1}$R-$G_{qi5(m)}$ also responded to baclofen (Fig. 7C).

Discussion

The present morphological and functional studies demonstrated that $\mu$OR and CB$_1$R form functional heterodimers. FRET and acceptor bleaching analysis demonstrated that $\mu$OR and CB$_1$R form heterodimers in BHK cells coexpressing $\mu$OR-Venus and CB$_1$R-Cerulean both in the plasma membrane and in cytosol. Coimmunoprecipitation followed by western blot analysis further supported the heterodimerization of $\mu$OR and CB$_1$R. Recent reports have shown that CB$_1$R and $\mu$OR form heterodimers as determined by optical bioluminescence resonance energy transfer (BRET) assay (7). Our results are in agreement with the above reports in that both receptors exist as heterodimers on the membrane or cytosol. In addition, we presented direct evidence for the first time that $\mu$OR and CB$_1$R form a functional heterodimer, by our electrophysiological experiments.

In the electrophysiological study, Xenopus oocytes coexpressing $G_{i/o}$-coupled $\mu$OR or CB$_1$R together with GIRK1/2 caused inward rectifying $K^+$ currents in response to the selective $\mu$OR agonist DAMGO and CB$_1$R agonist CP55,940, respectively, as reported previously (19, 22). The signals generated by stimulation of each receptor are transmitted to GIRK via $G_{\beta\gamma}$ subunits liberated from $G_{i/o}$ endogenously expressed in oocytes (10, 19). The $G_{qi5}$ is a chimeric $G_{q6}$ protein containing the last five amino acid residues from the C-terminus of $G_i$, which enables coupling of $G_{i/o}$-coupled receptors to the PLC signaling pathway (10, 11). By using fused receptors with $G_{qi5}$, we demonstrated that oocytes expressing either $\mu$OR-$G_{qi5}$ or CB$_1$R-$G_{qi5}$ alone activated PLC to subsequently elicit $Ca^{2+}$-activated $Cl^-$ currents in response to their specific agonists. Such a $G_{qi5}$-mediated signaling pathway induced by receptors that originally couple to $G_{i/o}$ proteins has been demonstrated in oocytes coexpressing other $G_{i/o}$-coupled receptors, namely muscarinic M$_2$, serotonin 5-HT$_1A$, somatostatin type 2, and GABA$_B$ receptors with coexpression of $G_{qi5}$, which was conducted in our laboratory (10, 11). The EC$_{50}$ values of these agonists were similar to those obtained in oocytes expressing the receptor together with GIRK1/2 and the receptor fused to $G_{qi5}$, suggesting that the efficacy is similar between the receptors/endogenous $G_{i/o}$ and the receptors/$G_{qi5}$ as reported previously (10). In oocytes coexpressing $\mu$OR and CB$_1$R, only one of which was fused to $G_{qi5}$, the agonist for the fused receptor elicited $Ca^{2+}$-activated $Cl^-$ currents, as expected. Moreover, both DAMGO and CP55,940, which are agonists for fused receptors and for non-fused receptors, could elicit $Ca^{2+}$-activated $Cl^-$ currents in those oocytes, which is a novel finding in our present study. One study using a three-dimensional structure model suggested that the homo- or heterodimeric signaling molecules of the GPCR-G protein complex were pentamers composed of two dimerized GPCRs with one trimeric G protein (23). Considered together, the present findings and those of the above study (23) suggest that $\mu$OR and CB$_1$R functionally interact on the plasma membrane and form a heterodimer and transmit the signal downstream through a common $G_{qi5}$ protein involved in the pentameric complex, but not through G proteins endogenously expressed in oocytes. Selective responsiveness in this interaction was revealed by the fact that oocytes coexpressing $\mu$OR + GABA$_{B2}$R + GABA$_{B1}$R-$G_{qi5}$ elicited $Cl^-$ currents only in response to baclofen but not DAMGO. Furthermore, $\mu$OR-$G_{qi5}$ + GABA$_{B2}$R + GABA$_{B1}$R responded to only DAMGO but not baclofen, indicating that $\mu$OR did not form a $\mu$OR/GABA$_B$R heterodimer. We also previously reported that $\mu$OR and GABA$_B$R did not form heterodimers with each other by FRET analysis (11).

The specific transactivating signal among the heterodimerized receptor complex was further confirmed by the PTX treatment experiments. In oocytes pretreated with PTX, most $G_{i/o}$ proteins were modified through their cysteine residue located at the 4th position from the C-terminus, and consequently, the signal from $G_{i/o}$-coupled GPCRs to $G_{i/o}$ was prevented (19 – 21). Even under PTX-pretreatment condition, $G_{qi5(m)}$ is insensitive so that signals from receptors can be transmitted through the $G_{qi5(m)}$. We clearly showed that both DAMGO and CP55,940 elicited $Ca^{2+}$-activated $Cl^-$ currents in PTX-pretreated oocytes coexpressing $\mu$OR + CB$_1$R-$G_{qi5(m)}$ or $\mu$OR-$G_{qi5(m)}$ + CB$_1$R. Taken together, our results suggest that $\mu$OR and CB$_1$R form a functional heterodimer on the plasma membrane.

Several studies reported that $\mu$OR forms homo- and heterodimeric structures with several types of receptors including opioid $\mu$OR and $\delta$OR, adrenergic $\beta$-R, and neurokinin NK1 receptor [see review (24)]. Furthermore, CB$_1$R has also been reported to form homo- and
heterodimers with CB1R itself, dopamine D2, and orexin OR1 receptors (25–27). In general, heterodimeric receptors show distinct pharmacological profiles compared with their parental receptors [see review (3)]. The synergy in the analgesic effects of opioids and cannabinoids can be attributed to a cross-talk between these two signaling pathways mediated by simultaneous activation of opioid and cannabinoid receptors. In rat nucleus accumbens core where μOR and CB1R are abundantly expressed, both receptors may physically associate and simultaneous stimulation of the receptors resulted in non-additive glutamate release and synergistic GABA release, suggesting the existence of functional μOR/CB1R heterodimers distinct from their parental receptors in the tissue (28). Electron microscopic studies on localization of CB1R and μOR in rat nucleus accumbens also supported coexistence of these receptors within the same neuron (29). The results reported by Rios et al. (7) and those of the present study showed that μOR formed a heterodimer with CB1R. In their report, simultaneous activation of μOR/CB1R heterodimer leads to a significant attenuation of the extracellular receptor-regulated kinase (ERK) activity compared to the responses seen upon activation of the individual receptor (7). Our results showed that separate application of each agonist elicited Ca2+-activated Cl− currents, and the sizes of the currents observed were almost similar to those obtained with oocytes expressing μOR-Gq5 or CB1R-Gq5 alone (data not shown). Although we did not conduct experiments to determine the effects of simultaneous application of both μOR and CB1R agonists on the cellular responses, we plan to do such experiments soon as part of our ongoing studies.

In summary, we demonstrated that μOR and CB1R form a functional heterodimer and may transmit a signal through a common G protein. It is important to clarify the physiological roles of μOR/CB1R heterodimer and to develop specific agonists/antagonists that act specifically on the dimerized forms of μOR/CB1R. Our novel approach using the heterologous expression system may be useful for such development. We propose that the electrophysiological method used in this study can be suitable for investigation and analysis of signals mediated through heterodimerized G protein–coupled receptors.

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